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ORIGINAL ARTICLE

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Triacylglycerol phase and 'intermediate' seed storage physiology: a study of *Cuphea carthagenensis*

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Abstract Seeds with 'intermediate' storage physiology store poorly under cold and dry conditions. We tested whether the poor shelf life can be attributed to triacylglycerol phase changes using *Cuphea carthagenensis* (Jacq.) seeds. Viability remained high when seeds were stored at 25°C, but was lost quickly when seeds were stored at 5°C. Deterioration was fastest in seeds with high (≥0.10 g g^{-1}) and low (0.01 g g^{-1}) water contents (g H_2O g dry $mass^{-1}$), and slowest in seeds containing 0.04 g g^{-1}. A 45°C treatment before imbibition restored germination of dry seeds by melting crystallized triacylglycerols. Here, we show that the rate of deterioration in *C. carthagenensis* seeds stored at 5°C correlated with the rate that triacylglycerols crystallized within the seeds. Lipid crystallization, measured using differential scanning calorimetry, occurred at 6°C for this species and was fastest for seeds stored at 5°C that had high and very low water contents, and slowest for seeds containing 0.04 g g^{-1}. Germination decreased to 50% (P50) when between 16 and 38% of the triacylglycerols crystallized; complete crystallization took from 10 to over 200 days depending on water content. Our results demonstrate interactions between water and triacylglycerols in seeds: (1) water content affects the propensity of triacylglycerols to crystallize and (2) hydration of seed containing crystallized triacylglycerols is lethal. We suggest that these interactions form the basis of the syndrome of damage experienced when seeds with intermediate storage physiologies are placed in long-term storage.

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Introduction

Triacylglycerols are believed to be important to the storage physiology and germination of seeds, though a mechanistic understanding of their role requires further investigation. The long-held presumption that seeds with high lipid contents age rapidly is disputed (Priestley 1986; Walters et al. 2005b). However, changes in the physical behavior of lipids during aging (Vertucci 1992; Walters et al. 2005a), the appearance of reaction byproducts suggesting the deesterification or peroxidation of lipids (Barclay and McKersie 1994; Smith and Berjak 1995), and a new understanding of amphiphile movement through polar and nonpolar domains of cells (Hoekstra and Golovina 2000) support the suggestion that triacylglycerols serve as a reservoir for substrates and protectants in aging reactions (Walters 1998). Triacylglycerol composition affects whether dry seeds are damaged when exposed to cryogenic temperatures (Stanwood 1987; Vertucci 1989). Coalescence of oil-bodies during seed imbibition is believed to influence the expression of seedling vigor (Huang 1992; Priestley 1986) and may be involved in seed recalcitrance (Leprince et al. 1998).

Recently, we showed that seeds are killed if exposed to water when their triacylglycerols are crystallized (Crane et al. 2003). Seeds prone to this type of damage generally have high proportions of saturated medium and long-chain fatty acids (≥C12) that are not fully melted at 25°C, the temperature typically used to imbibe seeds in the laboratory. Seeds may also be prone to this type of damage if stored under conditions that induce lipid crystallization and subsequently planted under field conditions that do not allow lipid crystals to melt. For example, seed bred for high oleic acid content may be damaged if they are stored in an unheated warehouse

during the winter in the northern US and Canada where temperatures commonly fall below −10°C and then planted in the spring before temperatures rise above 15°C (anecdotal from seed producers).

A survey of the literature suggests that seeds with the so-called "intermediate" storage physiology commonly have lipids with relatively high crystallization and melting temperatures (Crane et al. 2003). Seeds in this classification usually survive exposure to either low water contents or low temperatures, but do not survive when exposed to both stresses simultaneously (Ellis et al. 1990a, b). Because of their sensitivity to cold and dry conditions, seeds with intermediate storage physiologies are difficult to genebank, and hence the genetic diversity of species producing intermediate seeds is at risk.

Damage induced by a combination of low water content and low temperature is typically explained by phase changes water and water-soluble components of the cells (e.g., Crowe and Crowe 1992; Sun and Leopold 1994; Steponkus et al. 1995; Koster et al. 2000). However, the water contents and temperatures that induce damage in intermediate seeds (Sacandé et al. 2000; Ellis et al. 1991) do not correspond to those that result in phase changes of water or water-soluble components. Consequently, we have investigated interactions between water content, temperature, and phase properties of the non-aqueous components in seeds, namely the triacylglycerols.

The genus *Cuphea* (Lythraceae) is a convenient model for this study because many species produce seeds with storage lipids containing high levels of saturated medium-chain fatty acids (C_8–C_{14}) (Graham et al. 1981) that melt at temperatures ranging from −15 to 40°C (Crane et al. 2003). Crystallization of lipids in *Cuphea* seeds with high lauric (C_{12}) or myristic (C_{14}) acids was induced by cooling to −18°C, and a 10-min heat pulse at 45°C prior to imbibition was necessary to melt the lipids and allow the seeds to germinate normally (Crane et al. 2003). In this paper, we show that germination of *C. carthagenensis* seeds was reduced after brief storage at 5°C, and that the loss of viability was exacerbated under extremely dry or moderately moist conditions, RH < 12% or ≥75%, respectively. We show that this pattern of germination loss, symptomatic of seeds classified as having intermediate storage physiology or of seeds considered to be sensitive to chilling, can be attributed to the temperature and kinetics of triacylglycerol crystallization.

Materials and methods

Cuphea carthagenensis seeds (accession 'Ames 17845', which originated in Chiapas, Mexico) used in this research contained 32% lipid as determined by quantitative analysis using a modified Bligh and Dyer technique (1959). Extracted lipid consisted of 61% lauric (C_{12}) and 22% myristic (C_{14}) acids in the triacylglycerol fraction and 22% palmitic (C_{16}), 20% oleic ($C_{18:1}$) and 33% linoleic ($C_{18:2}$) acids in the polar lipid fraction (Crane et al. 2003). Fresh seeds of this accession were obtained from plants grown in greenhouses at the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA, USA in 2001 and shipped to the National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO, USA. Seeds of *C. carthagenensis* do not exhibit dormancy (Graham 1989) and 85% of seeds from the lot germinated when tested a few days after their arrival in Fort Collins using the procedures described below.

The seeds were pretreated at 45°C for 1 h to ensure that all lipids were fully melted at the onset of the experiment (Crane et al. 2003). Prior to storage experiments, seed water content was adjusted to water contents between 0.008 and 0.2 g H_2O g dry mass^{-1} (g g^{-1}) by placing seeds in ten different RH chambers at room temperature (23 ± 1°C) for 10 days. Relative humidity, controlled by saturated salt solutions, ranged from 1 to 90%. Seeds were then sealed into small foil laminate bags (Barrier Foil Products Co. Denton, Manchester, UK) and placed at 5°C. Seed water content was measured gravimetrically at each sampling time, with dry masses determined after heating samples for 96 h at 90°C. Water content is expressed on a dry mass basis and represents the average of three replicates measured from each bag at each sampling time.

Viability of seeds was assessed from periodic germination assays. Prior to the hydration treatments, half of the seeds were placed at 45°C for 1 h to fully melt triacylglycerols that might have crystallized during storage at 5°C. All seeds were then slowly hydrated to prevent imbibitional damage by placing them in a plastic box above distilled water for 18 h. Seeds were then placed on damp blotter paper in Petri dishes in an incubator held at 25°C with a 16–8 h light–dark cycle for up to 6 weeks. Germination was scored when both the radicle and hypocotyl emerged. Data showing changes in viability with time were fit to Avrami kinetics (Walters et al. 2004, 2005b) and aging rates and times to 50% germination (P50) were calculated from the time coefficients (see Walters et al. 2004, 2005b for calculation details).

Thermal behavior of lipids in the seeds of *C. carthagenensis* was measured using a Perkin–Elmer differential scanning calorimeter (DSC7; Norwalk, CT, USA) calibrated for temperature with methylene chloride (−95°C) and indium (156.6°C), and for energy with indium (28.54 J g^{-1}; Vertucci 1992). Samples containing approximately 4 mg of whole seed were hermetically sealed into Perkin–Elmer volatile sample pans and then loaded into the DSC which had been prechilled to 10°C. Heat flow was recorded as samples were warmed from 10 to 50°C (first warming run), cooled from 50 to −100°C, and finally warmed again to 50°C (second warming run). All cooling and warming was done at 10°C min^{-1} and the samples were held 1 min at the end of each cooling or warming run. Onset temperatures of the lipid transitions were calculated using Perkin–Elmer software from the intersection of the baseline and the

tangent to the steepest part of the transition peak. The amount of lipid that crystallized or melted was quantified by the enthalpy of the transitions, which was calculated from the area encompassed by the transition peaks using Perkin–Elmer software. The amount of lipid that crystallized during storage at 5°C was inferred from the enthalpy of melting transitions recorded when seeds were warmed in the first DSC scan from 10 to 50°C. Total lipid crystallization was calculated from the melting enthalpy recorded when seeds were warmed to 50°C a second time, after being cooled to −100°C. Sample dry mass was measured after heating samples for 96 h at 90°C and enthalpy is expressed on a dry mass basis. Calorimetric data were collected from three replicates at each water content and sampling interval. Rate of lipid crystallization was calculated from time courses of the increase in melting enthalpy in seeds stored at 5°C, which appeared to follow zero-order (linear) kinetics initially and first-order (logarithmic) kinetics after 10 days of storage. Time coefficients were calculated for the two stages and used to quantify initial crystallization rates and the time required for 50% of lipids to crystallize (C50).

Results

Germination percentage of *C. carthagenensis* seeds stored at 5°C and imbibed without first receiving a 45°C treatment decreased from 95 to 0% within 100 days for all hydration treatments (Fig. 1). Germination loss was fastest for seeds stored at the highest water contents and decreased when seeds were dried to a water content of 0.04 g g^{-1}. Extreme drying, to water contents as low as 0.008 g g^{-1}, increased the rate of deterioration (Fig. 1). Trends were different for seeds stored at 25°C and water contents < 0.07 g g^{-1}: germination remained high throughout the reporting period (data for 0.07 g g^{-1} are given in Fig. 1).

A simple warming treatment to 45°C before prehydration dramatically changed the aging kinetics of *C. carthagenensis* seeds stored at 5°C for seeds containing ≤ 0.1 g g^{-1} (Fig. 2). After warming, seeds containing 0.07 g g^{-1} showed no evidence of deterioration even after 330 days of storage, and average germination percentage was constant at 94% (standard deviation = 6%; Fig. 2).

The time taken for germination to decrease to 50% (P50, see Walters et al. 2004, 2005b for calculation details) shows that the 45°C treatment had no substantial effect on P50 for seeds stored at 0.2 g g^{-1} (P50 = 1.4 and 1.1 days, without and with the 45°C pulse, respectively) or for seeds containing 0.13 g g^{-1} (P50 = 2.5 and 5.8 days, without and with the 45°C pulse, respectively). However, P50 increased for seeds stored at 5°C by drying seeds to water contents < 0.13 g g^{-1} and by giving them the 45°C pulse (Fig. 3). The P50 for seeds containing 0.10 g g^{-1} increased from 3.6 days if no heat pulse was given to 107 days (Fig. 3, filled circles) if a heat pulse was given. The P50 increased to a maximum of about 50 days for seeds dried to water contents between 0.04 and 0.05 g g^{-1} and not receiving the 45°C treatment, but decreased when the storage water content

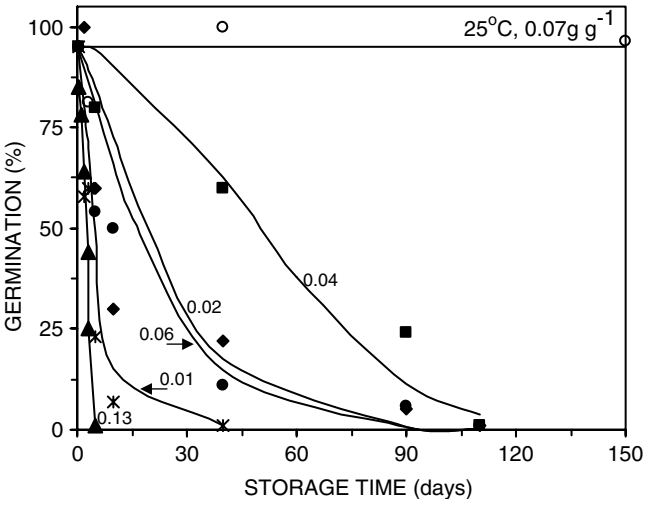

Fig. 1 Germination of *C. carthagenensis* seeds stored at 5°C and different water contents (*filled symbols*). Water contents are indicated by *numerals* near curves and data are representative of time courses collected for additional water contents. After the storage time, seeds were brought to room temperature, prehydrated in water vapor overnight and then imbibed at 25°C. Germination data for seeds stored for the same time period at 25°C and 0.07 g g^{-1} are also provided (*open symbols*). Curves were calculated by fitting time course data to Avrami kinetics (Walters et al. 2004, 2005b)

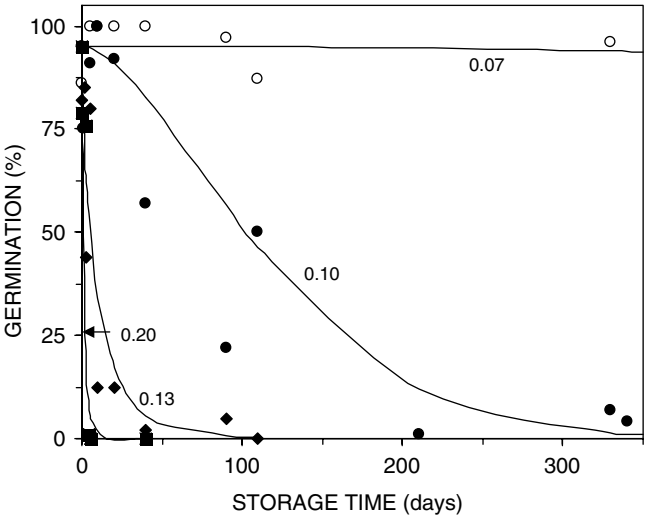

Fig. 2 Germination of *C. carthagenensis* seeds stored at 5°C and different water contents and receiving a 1-h 45°C heat pulse after storage. Treatments of these seeds were identical to those presented in Fig. 1 except that these seeds were warmed to 45°C, rather than room temperature, before being prehydrated in water vapor overnight. Seeds containing 0.07 g H_2O g dm^{-1} (*open symbols*) or less water did not show appreciable deterioration within 300 days of storage. Curves were calculated by fitting time course data to Avrami kinetics (Walters et al. 2004, 2005b)

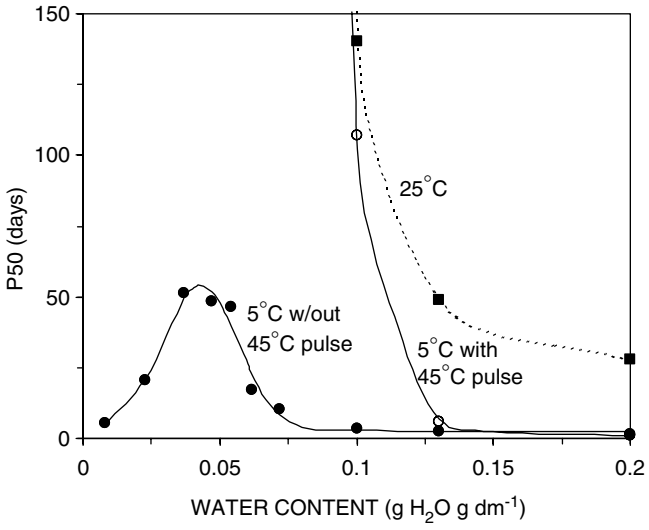

Fig. 3 The time for germination to decrease to 50% (P50) as a function of water content and storage temperature. Storage temperature at 5°C is represented by *circles* (*open* and *filled* for seeds receiving and not receiving the 45°C heat treatment after storage) and at 25°C is represented by *squares*. Values for P50 were calculated from Avrami curves fit to data provided in Figs. 1, 2 and data collected for seeds stored at additional water contents. Seeds containing less than 0.10 g g^{-1} that were stored at 25°C or at 5°C and followed by the heat pulse had P50 values near or greater than 625 days. The curve for 5°C (no heat pulse) serves as an aid to the eye and was calculated by fitting P50 versus water content data to a Gaussian function using SigmaPlot software (Systat Software Inc., Point Richmond, CA, USA)

was reduced further. Greater longevity was exhibited by seeds stored at 25°C for all water contents studied.

We also investigated the interactions of seed water content and temperature on lipid melting and crystallization behavior. Thermograms of whole *C. carthagenensis* seeds warmed from 10 to 50°C revealed an

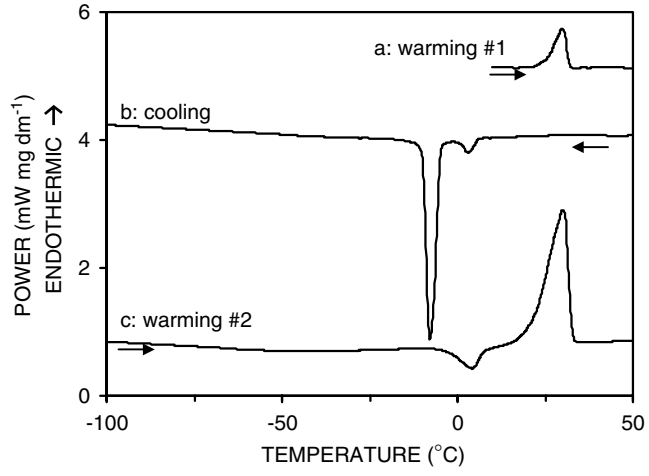

Fig. 4 a–c Representative DSC scans showing the experimental protocol used to quantify lipid crystallization during storage at 5°C and upon exposure to −100°C. Seeds held at 5°C were loaded into a prechilled DSC and warmed from 10 to 50°C (**a**), then cooled to −100°C (**b**) and then re-warmed from −100 to 50°C (**c**)

endothermic event with onset at 25°C, reflecting the melt of lipids crystallized during 5°C storage. Figure 4 presents representative scans to describe experiments. Figure 4a shows representative thermograms of seeds held at 5°C, but not exposed to lower temperatures. During cooling to −100°C, two exothermic events, indicative of lipid crystallization, were observed at 6 and −5°C (Fig. 4b). Upon warming from −100°C, an exothermic event at about 5°C, representing the recrystallization of lipids, was followed by a melting endotherm at about 22°C (Fig. 4c). This endotherm was always larger than the melting endotherm observed during the first warming run (Fig. 4a) and occurred at a slightly lower temperature (Fig. 5a).

The temperatures at which endo- or exothermic events were recorded did not change significantly with water content or storage time over the 120-day storage period at 5°C. Onset temperature in Fig. 5 is expressed as a function of water content and was 25.6 ± 1.3°C, according to data averaged among storage times (squares in Fig. 5a). Onset temperature for melting events was about 3°C lower in seeds that were cooled to −100°C at 10°C min^{-1} (circles in Fig. 5a) and averaged 21.6 ± 1.1°C (see Fig. 4c for a representative DSC scan). The lower temperature of melting events from the second warming run probably reflects other molecules mixing with triacylglycerols as a result of the first warming. Two exothermic events at 6.2 ± 0.8°C and −4.5 ± 0.6°C were consistently observed during cooling (see Fig. 4b) and did not significantly change with storage time or water content (Fig. 5b).

The endo- and exothermic events recorded in DSC traces are attributed to triacylglycerol transitions. The size and temperature of the endothermic events are consistent with lipids containing high amounts of lauric and myristic acids (Small 1988; Crane et al. 2003). The exothermic events in Fig. 4b, c that occur near 0°C cannot be attributed to pure water since water freezing transitions are severely limited in seeds containing less than 0.2 g g^{-1} (e.g., Vertucci 1990). Transitions from the polar lipid fraction are ruled out because the observed temperature of the melting peak (~35°C; Fig. 4) is greater than is expected for polar lipids from *C. carthagenensis* (~1°C; Crane et al. 2003) and is also not affected by water content (Fig. 5). The exothermic event observed upon warming in Fig. 4c is commonly observed in rapidly cooled triacylglycerols, demonstrating that the 10°C min^{-1} cooling used in DSC studies was too fast to permit the molecular reorganizations necessary for lipids to fully crystallize (Wendlant 1974; Small 1988).

The enthalpy of the melting endotherm recorded in seeds that were cooled to −100°C (see Fig. 4c) was 39.5 ± 0.5 mJ mg dry $mass^{-1}$, with no significant differences observed for seeds stored at 5°C for different storage times or at different water contents, although there was a slight trend toward increasing enthalpy with increasing water content (data not shown). The crystallization enthalpy calculated as the sum of enthalpies

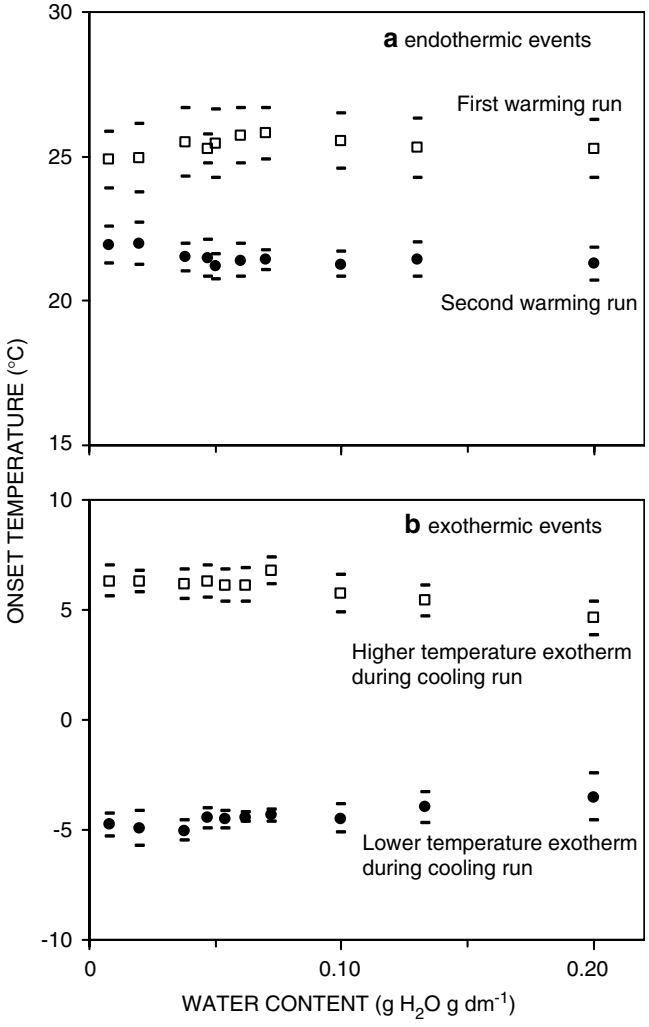

Fig. 5 **a**, **b** Temperature of lipid melting (**a**) and crystallization events (**b**) in *C. carthagenensis* seeds as a function of the water content of the seeds. The onset temperature of the transition is reported and represents the average value (*points*) and standard deviation (*error bars*) for seeds stored at 5°C for 10–120 days (see representative scans in Fig. 6). In **a**, data points represent onset temperatures for seeds following storage at 5°C (*squares*, representative scan in Fig. 4a) and following cooling to −100°C (*circles*, representative scan in Fig. 4c). In **b**, data points represent onset temperatures of the small (*open squares*) and large (*filled circles*) exothermic events recorded in Fig. 4b

measured from exotherms during both cooling and heating was 36.9 ± 0.8 mJ mg dry mass^{-1} (data not shown), and was slightly less than the enthalpy measured for melting because of limitations of the technique and instrumentation (Wendlant 1974). The melting enthalpy expected for a triacylglycerol mixture containing 61% lauric, 22% myristic, and lesser amounts of other fatty acids, representing the composition of this accession of *Cuphea* (Crane et al. 2003), is 187 J g lipid^{-1} according a weighted average of the enthalpies for pure triacylglycerols (i.e., trilaurin, trimyristin, tripalmitin, triolean, etc.; Small 1988). Approximately 32% of the dry mass of this seed is lipid, while 2% of the dry mass is polar lipid. Assuming that the remaining 30% of the dry mass is triacylglycerol, a melting enthalpy of 56 J g dm^{-1} is expected. The measured value is lower, 39.5 J g dm^{-1}, and suggests that only 70% of the lipids crystallized or that proteins and other non-lipid constituents coeluted within the nonpolar fraction (Walters et al. 2005a).

The enthalpy of the melting transition for seeds stored at 5°C (measured in initial DSC scans from 10 to 50°C; see Fig. 4a) approached 39.5 J g dm^{-1} as storage time increased, becoming similar to the average enthalpy measured after seeds had been cooled to −100°C (measured in the second DSC scan from −100 to 50°C; see Figs. 4a, 6, 7). Hardly apparent initially (see thermograms for 0 time in storage in Fig. 6a–c), the peak was discernable in seeds that were placed at 5°C for about 5 days, and became progressively larger until a maximum enthalpy of between 39 and 40 J g dm^{-1} was achieved (enthalpy values provided adjacent to thermograms in Fig. 6). Initial rates of crystallization, calculated from linear regressions of enthalpy versus storage time, and time to 50% crystallization (C50), calculated with the assumption that final crystallization enthalpy = 40 J g dm^{-1}, varied with seed water content. The highest crystallization rates and lowest C50s were observed in the wettest (≥0.10 g g^{-1}) and driest (0.01 g g^{-1}) seeds (Figs. 7, 8). Crystallization was slowest in seeds containing 0.04 g H_2O g dm^{-1} (Fig. 8).

Water sorption isotherms for this accession of *C. carthagenensis* are given for 5 and 25°C in Fig. 9. These isotherms have temperature–water content–RH relationships typical of orthodox seeds. The critical water contents for maximum longevity (P50) and minimum crystallization rate (C50) coincide at 0.04 g g^{-1} (compare Figs. 3, 8) which corresponds to about 12% RH at 5°C according to Fig. 9. The water content at which seeds are irreversibly damaged by exposure to low temperature is above 0.10 g g^{-1} and corresponds to about 75% RH (Figs. 1–3). A significant correlation exists between crystallization rate (C50) and longevity (P50; Fig. 10, $r^2 = 0.89$; $P < 0.01$). About 28–38% of the lipid crystallized at times corresponding to P50 (enthalpies of 11–15 J g dm^{-1} according to Fig. 7) for seeds stored at 5°C and water contents ≤0.10 g g^{-1}, and about 16–25% of the lipid crystallized by P50 in seeds stored at water contents ≥0.13 g g^{-1}.

Discussion

Seeds of *C. carthagenensis* exhibit storage behavior typical of seeds identified to have intermediate storage physiologies (Hong et al.1996; Ellis et al. 1990a, b): they survive extreme desiccation, but deteriorate relatively quickly during low-temperature storage (Figs. 1, 2; Crane et al. 2003). The loss of viability of intermediate seeds during dry storage has been attributed to phase transitions or amphiphile partitioning within the aqueous portion of the seed (Sacandé et al. 1998, 2000, 2001;

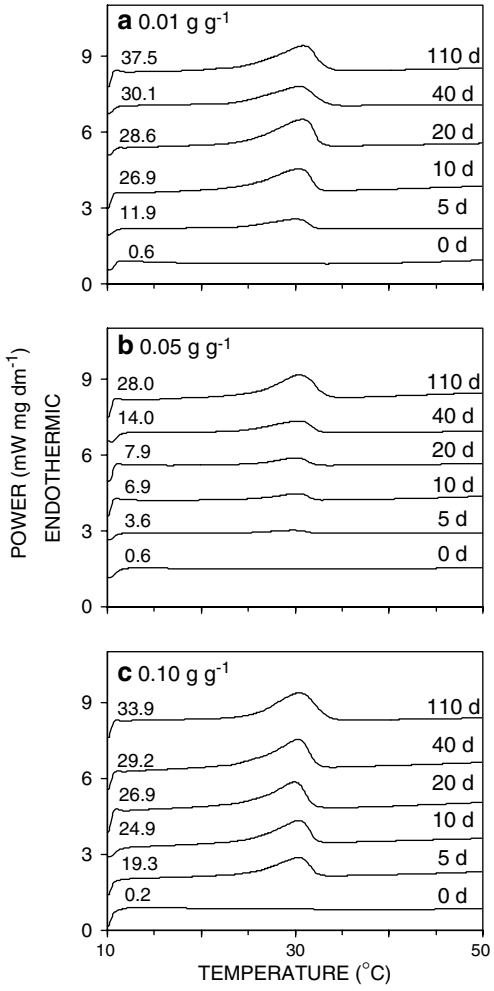

Fig. 6 a–c DSC scans of C. *carthagenensis* seeds stored at 5°C for the indicated time during initial heating to 50°C. A family of scans is given for seeds containing different water contents (*top panel* 0.01 g g^{-1}, *middle panel* 0.05 g g^{-1} and *bottom panel* 0.10 g g^{-1}), which are representative of scans for other water contents and storage times. DSC methods follow those described for Fig. 4a. Values for power (ordinate) and enthalpy of the melt during the first warming run (to the left of the scan) are expressed in terms of the dry mass of the sample

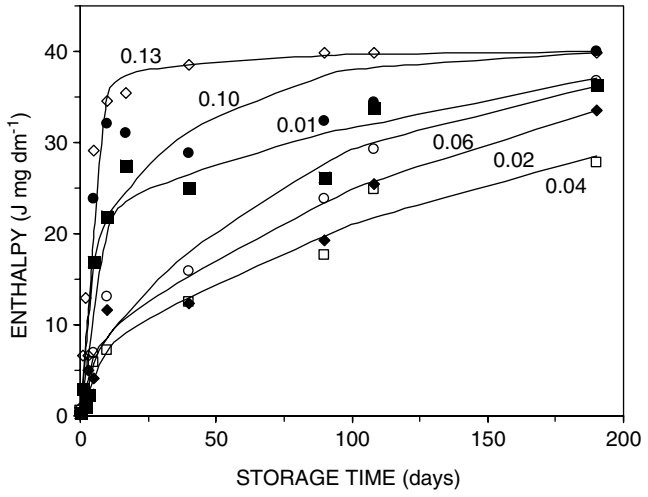

Fig. 7 The change in lipid melting enthalpy during the initial warming of seeds from 10 to 50°C after seeds had been stored for up to 200 days at 5°C and a range of water contents. Data are taken from enthalpy values in Fig. 6 and from similar data obtained for other water contents. Curves are calculated assuming zero-order kinetics (linear) for the first 10 days and first-order kinetics (logarithmic increase to maximum of 40 J g dm^{-1}) for time > 10 days

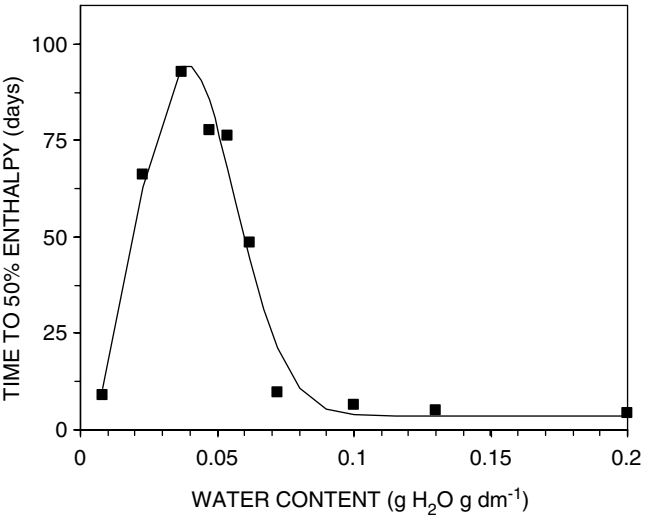

Fig. 8 Effect of water content during storage at 5°C on crystallization rate of lipids in *C. carthagenensis* seeds. Crystallization rate is expressed as the storage time when enthalpy of the lipid melting transition was half of the total enthalpy (40 J g dm^{-1} of the seed) (C50), and was calculated from the time courses described in Fig. 7. The curve serves as an aid to the eye, calculated by fitting C50 versus water content data to a Gaussian function using SigmaPlot software

Neya et al. 2004). Most recently, the intermediate physiology of neem seeds (*Azadirachta indica)* was attributed to imbibitional stress that could be alleviated by hydrating seeds in warm water (Neya et al. 2004), and we show here that just a dry heat treatment is effective for *Cuphea* (Crane et al. 2003; Fig. 3). Symptoms of intermediate physiology have also been intermingled with concepts of dormancy and dry after-ripening (e.g., King et al. 1981; Wood et al. 2000; Merritt et al. 2005). Stimulating germination of dried seeds using a heat treatment is a recurrent theme for seeds with dormant or intermediate physiologies that originate in warm climates. The heat treatment becomes ineffective after seeds are hydrated to > 0.10 g g^{-1} (or > 70% RH; Figs. 2, 3). Thus, susceptible seeds are irreversibly damaged by hydrated storage under cool conditions and are known to be "chilling sensitive" (Ibanez 1964; Berjak et al. 1995; Sacandé et al. 1998; Pammenter and Berjak 1999; Danthu et al. 2000).

Damage to *Cuphea* seeds can be induced and ameliorated by simple temperature fluctuations. The reversibility of the reactions implies a physical change such as a phase transition. In a previous paper, we

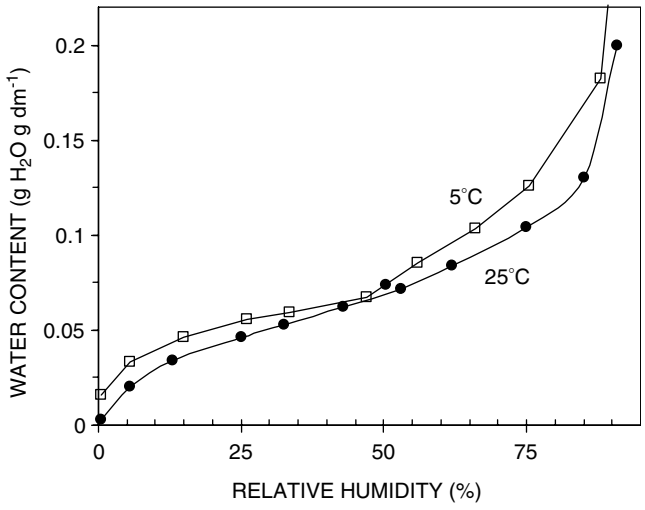

Fig. 9 Water sorption isotherms of *C. carthagenensis* seeds constructed at 5 and 25°C. Relative humidity was controlled by saturated salt solutions over which seeds were incubated until their fresh mass was constant

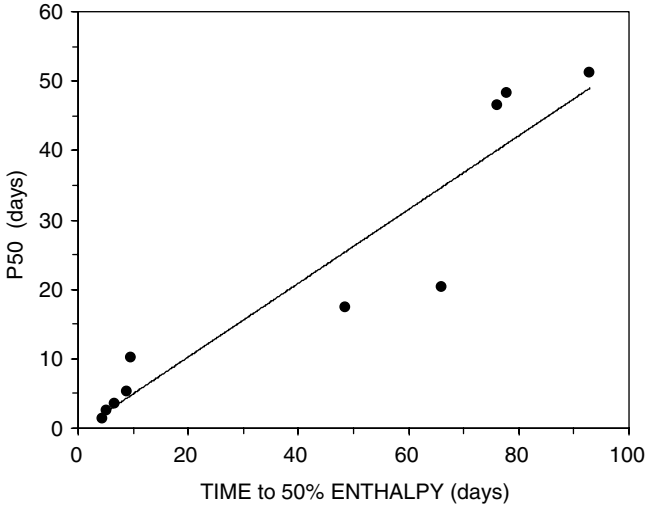

Fig. 10 The relationship between the rate of lipid crystallization (time to 50% enthalpy, C50) and the loss of viability (P50) of *C. carthagenensis* seeds stored at 5°C. Values for P50 are from Fig. 3 and for lipid crystallization rate are from Fig. 8. Germination data are for seeds that did not receive a 45°C heat pulse before hydration. The curve is the least squares fitted line and has an $r^2 = 0.89$ ($P < 0.01$)

showed a cause–effect relationship between triacylglycerol phase and damage in seeds of *Cuphea* species containing different fatty acids (Crane et al. 2003), and in this paper we show a correlation between the kinetics of triacylglycerol crystallization and deterioration rate during 5°C storage of the test species, *C. carthagenensis* (Fig. 9). The storage temperature is too high to induce crystallization of water or polar lipids and the constant storage temperature rules out effects of aqueous glasses. Phase changes in the triacylglycerol fraction are not damaging per se. Based on our experience with cryogenic storage, we know that seeds from many species can be repeatedly exposed to temperatures that induce lipid crystallization providing that they are not cooled too rapidly (e.g., Stanwood 1987; Vertucci 1989; Walters et al. 2004). The lipids within seeds from most of the species in our genebank melt at subzero temperatures and, until now, the occasion of imbibing seeds containing crystallized lipids has not arisen.

While crystallization and melting of triacylglycerols appear to be completely reversible, we report damage if lipids are crystalline when a sufficient amount of water is available. The damage is expressed during imbibition if seeds are stored dry and during storage if seeds contain water contents ≥0.10 g g^{-1}. The symptoms of damage upon imbibition reported here are different than those classically described as imbibitional stress (e.g., Hobbs and Obendorf 1972; Simon 1974; Bramlage et al. 1978; Hoekstra et al. 1999): drying *Cuphea* seeds to 0.04 g g^{-1} retards damage and increases P50 (Fig. 2), and slowly hydrating *Cuphea* seeds does not prevent damage (Crane et al. 2003, all treatments in this report). However, damage during hydrated storage as reported here appears to be analogous to early discussions of chilling injury (e.g., Lyons et al. 1979; Wu and Browse 1995), except that we suggest the triacylglycerol, rather than the membrane fraction, is the site of damage, a possibility that was first introduced in the 1960s (Ibanez 1964).

It is not immediately clear why the interaction of water with crystallized storage lipids is lethal. Our best speculation builds on recent ideas of partitioning of amphiphilic molecules and surface-active agents into hydrophilic or hydrophobic regions depending on water content (Golovina et al. 1998; Hoekstra et al. 1999). Within the triacylglycerol fraction, amphiphilic molecules may catalyze or inhibit crystallization reactions (see Walters et al. 2005a), thereby explaining differences in crystallization rate, but not total crystallization, with seed water content (Figs. 6, 7, 8). When the lipid crystallizes, these molecules will be trapped within or excluded from triacylglycerol regions, depending on the water-mediated partitioning behavior, and lose their surface-active functions. Oleosins are an interesting candidate for this model, since they are located at the lipid body–cytoplasm interface and are believed to regulate surface area of lipid bodies (Huang 1992). Massive coalescence of lipid bodies was observed when seeds containing triacylglycerols with high melting temperatures were imbibed at ambient temperatures (Leprince et al. 1998; Neya et al. 2004), and absent or dysfunctional oleosins were implicated (Leprince et al. 1998). Our speculation is consistent with Leprince and colleagues' hypothesis that oleosins are required to maintain the lipid body–cytoplasm interface, and thereby prevent fusion of lipid bodies in hydrated cells. Currently, we are testing whether lipid bodies coalesce in *Cuphea* seeds in response to temperature and water content treatments. In the future, we will measure the

partitioning of oleosins with water content and temperature.

The most distinguishing feature of seeds classified as having 'intermediate' storage physiology is their relatively rapid aging under cold and dry storage conditions, and it has been suggested that these seeds fall between orthodox and recalcitrant seeds on a continuum of desiccation tolerance or longevity (e.g., Walters et al. 2002). Our studies using *Cuphea* suggest that these seeds express sensitivity to temperature rather than desiccation, and that the temperature range and accumulation of damage correspond to triacylglycerol phase behavior and crystallization rate. Damage is manifested when crystallized lipids are exposed to water, implying that imbibitional stress and chilling during hydrated storage cause the same injury to the seeds.

Storage behavior of *C. carthagenensis* seeds fits the description of seeds classified as having intermediate storage physiology. The damaging effect of low-temperature storage and the apparently rapid aging correspond to crystallization of triacylglycerols and can be reversed by melting triacylglycerols in seeds before they are imbibed. We suggest that the interaction between water and triacylglycerols forms the basis for the syndrome of damage experienced when seeds with intermediate storage physiologies are placed into long-term storage.

Our results indicate that genebanking seeds with intermediate physiology, caused by lipids that melt at high temperatures, is eminently feasible. Genebankers should follow protocols outlined for orthodox seeds, but make particular efforts to keep seeds with crystallized lipids dry. However, a cautionary note is warranted: *Cuphea* seeds stored in our lab at 5 and −5°C for about 2 years may be showing unexpectedly early signs of deterioration that are not observed in seeds stored at 15, −18, −80 or −150°C (data not shown). To avoid these apparent anomalies, we recommend storing seeds at temperatures outside the range for which lipid crystallization reactions are observed.

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